

Vaccination of Balb/c mice against enteroviral mediated myocarditis

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A non-virulent strain of Coxsackie B3 (CB3) virus was used to produce a subunit vaccine. It contains the capsid proteins VP1, 2, 3 and probably 4 and can be made RNA-free. It is based on the ISCOM technology ensuring non-toxic properties and good adjuvant effect. Vaccinated animals at doses above 16 ng were completely protected from mortality when challenged with a myocarditic strain of CB3. Histologically no inflammatory lesions were found in the heart. This was corroborated using immune histological techniques with monoclonal antibodies against lymphocyte subsets. Even at a dose of 0.16 ng a delayed mortality was observed. Neutralizing antibody titres rose to 512, thus ensuring a circulating level well above that considered protective. It is suggested that vaccination might be a possible way of prophylaxis for myocarditis and even dilated cardiomyopathy, the latter presently being the chief cause of heart transplantation. By persistence or triggering of autoimmune phenomena Coxsackie virus is incriminated as the first step in pathogenesis.

Keywords: Coxsackie B3; enteroviral-mediated myocarditis; subunit vaccine; capsid proteins

Introduction

Enteroviruses encompass altogether about 70 serotypes of the subgenus Coxsackie types A and B, ECHO and polioviruses. Other picornaviruses are hepatitis A virus and about 100 serotypes of rhinoviruses¹. The very similar structure at the genetic level of all these viruses has recently been demonstrated by the advent of RNA sequencing methods². The morbidity and complications caused by these infectious agents are probably underestimated due to cumbersome or the absence of diagnostic procedures.

Recently renewed evidence has accumulated to indicate that the Coxsackie B (CB) viruses may trigger 15–76% of cases with juvenile onset diabetes^{3–5}. The life-long suffering of these patients is well known to everyone working in the medical field. Also CB viruses are considered to be the predominating viruses causing myocarditis in man⁶. Some authors claim that acute myocarditis in man may progress to dilated cardiomyopathy (DCM)⁷. Conversely, there is suspicion that a large proportion of the DCM cases are caused by CB viruses, starting as a subclinical myocarditis. Such a course of events has been described in an experimental model⁸. This is reinforced by recent findings of Coxsackie viral RNA, demonstrated by RNA hybridization techniques, in endomyocardial biopsies from DCM patients⁹. DCM accounted for 51% of the 2577 patients who received a heart transplant up till the end of 1986¹⁰.

Generally, vaccines for viral disease are based on either attenuated virus strains, killed virus or components of virus (subunit vaccines). In this study we have used an

attenuated viral strain and a subunit vaccine based on the ISCOM technology¹¹. By immunization of mice by the use of a CB3 strain in a model system we have been able to induce full protection against a virulent CB3 strain causing lethal myocarditis in mice.

Materials and methods

Virus strains and cells

Coxsackie B3 attenuated strain called RD was obtained by Dr Lindberg, Department of Medical Genetics, Biomedical Centre, Uppsala and was originally produced in Dr Crowell's laboratory, Philadelphia, USA. It was grown on a rhabdomyosarcoma cell line in a 2 litre culture using RPMI 1690 medium supplemented with 5% fetal calf serum (FCS) and antibiotics. The virus suspension from a 2 litre culture was centrifuged on a caesium gradient to yield purified virus and procapsids¹². This material was dissolved in 0.05 M phosphate buffer, 0.1 M NaCl, pH 7.2 (PBS) to a final concentration of 3 mg protein ml⁻¹. The protein concentration was measured by the method of Bradford¹³.

A myocarditic Coxsackie B3 strain was obtained from Dr S. Huber, Burlington, Vermont. It is usually referred to as the 'Woodruff' strain, from the late originator. It was propagated in HeLa cells. The cells were grown in Eagles MEM supplemented with 5% FCS and antibiotics.

Virus titres were determined on HeLa cells as plaque forming units (p.f.u.) and appropriate dilutions were made. Stock solutions were stored deep frozen (–20°C).

Animals

Balb/c CUM mice from Cumberland farms, Clinton, TN, USA, were kept in a breeding colony. Food and water were supplied *ad libitum*. Only male mice aged 4–10 weeks at the start of the experiment were used. Temperature was controlled at 23±1°C and a 12 h light/dark cycle was maintained.

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Infection of animals

Mice were infected intraperitoneally (i.p.) using small bore fixed cannula syringes (1 ml standard 'insulin' syringe). Infection was done with 5×10^4 p.f.u. in 0.2 or 0.5 ml physiological buffered saline.

Subunit vaccine preparation

The preparation of ISCOM is a modification of the original procedure described by Morein *et al.*¹¹. To 2 ml of caesium gradient purified virus suspension, containing 6 mg protein, Tris NaCl buffer was added a final concentration of 0.1 M to adjust to pH 9. Further urea was added to 6 M, EDTA to 0.002 M and dithiothreitol (DTT) to 0.002 M. To this mixture 0.7 mg lipid was added from a stock solution containing 10 mg cholesterol and 10 mg phosphatidylcholine in H₂O with 10% MEGA-10 prepared according to Hildreth¹⁴.

Further MEGA-10 to 1% and QuilA to a concentration of 0.1% were added. The mixture was left for 50 min at 37°C, then in room temperature for 50 min and then dialysed for 3 days against a buffer of 0.1 M Tris, 1 M urea, 0.002 M EDTA and 0.002 M DTT. After that the dialysis buffer was changed to 0.1 M Tris NaCl, 0.002 M EDTA. The formation of ISCOM was verified by electron microscopy. The mixture was first cleared from precipitate by centrifugation for 10 min at 1000g and then layered on 10% sucrose in PBS and centrifuged for 19 h at 39 000 rev. min⁻¹ at 15°C. The pellet was dissolved in 500 µl PBS and the formation of ISCOM was verified by electron microscopy. The protein content was determined to be 260 µg ml⁻¹. Neomycine sulphate 50 µg ml⁻¹ was added. Mice were injected i.p. and s.c. with different dilutions from this stock solution.

Vaccination scheme

RD virus corresponding to 5×10^4 p.f.u. was injected i.p. in group 1. Group 2 and 3 received 0.65 µg of the ISCOM vaccine either i.p. or s.c. A booster dose was applied after at least 3 weeks. Mice were then rested for at least 2 weeks. The Woodruff myocarditis CB3 variant (5×10^4 p.f.u.) was then injected i.p. into the vaccinated animals as well as a control group. The dose-response relationship was tested in a separate experiment. In each group 10–20 animals were boosted twice.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

A Pharmacia gel electrophoresis apparatus (PHAST-system) was used to analyse the ISCOM vaccine preparation on a 10–15% gradient gel. Staining was by Coomassie Brilliant Blue, using the standard automatic program. Pharmacia calibration kits for molecular weight determination using electrophoresis with the following reference proteins (in kDa) were used. Low molecular weight: α-lactalbumin (14.4), trypsin inhibitor (20.1), carbonic anhydrase (30), ovalbumin (43), albumin (67), phosphorylase b (94). High molecular weight: albumin (67), lactate dehydrogenase (36), catalase (60), ferritin (18.5, 220), thyroglobulin (330).

Histology

Animals were killed at day 8 or 12 after ether anaesthesia. Blood was withdrawn, the heart was cut transversally into two pieces—one for formalin and the

other for freezing. Standard procedures for paraffin bedding and microtome section were performed for hematoxylin-eosin staining. The frozen part was sectioned in a cryostat and stored at -70°C until use. Immune histology using monoclonal lymphocyte markers was performed as described¹⁵.

Virus neutralization

GMK cells were used as previously described for neutralization titre determinations⁴ of pooled sera.

Results

Characterization

The electron micrograph of the vaccine preparation (Figure 1), shows the typical cage-like ISCOM structure¹¹. In SDS-PAGE three bands were detected corresponding to the molecular weights of VP1, 2 and 3, i.e. 24, 27 and 30 kDa respectively (Figure 2). VP4 (7 kDa) was not visualized in the same gel.

Protection

Vaccinations of animals were done as delineated in the Materials and methods section. Two weeks after boosting and resting, mice were challenged with the myocarditis CBV3 strain (Woodruff). Normally mice start to get



Figure 1 The ISCOM vaccine preparation electron microscope analysis at a magnification of 75 000

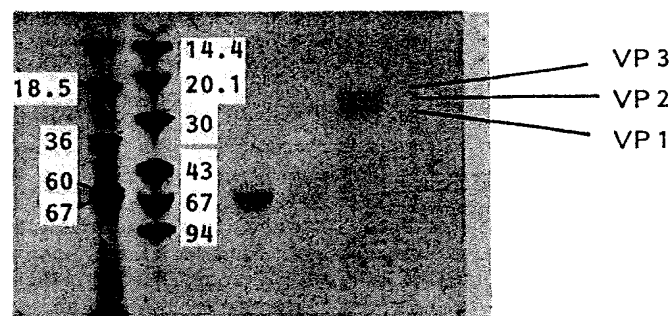
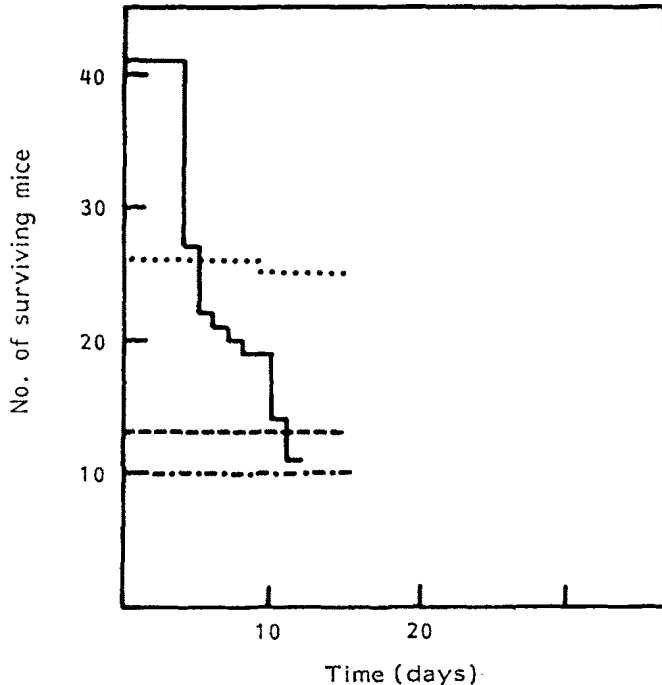


Figure 2 SDS-gel electrophoresis of ISCOM-CB3-RD vaccine preparation. Staining was with Coomassie Brilliant Blue. Lane 1 reference proteins (HMW), lane 2 reference proteins LMW, lane 3 cell culture medium, lane 4 vaccine preparation. M_r of markers given in kDa

Table 1 Experimental mortality on day 12

	Mortality (%)	Neutralization titre
Woodruff CB3	78 (32/41)	≤32
Woodruff CB3+RD CB3	4 (1/26)	64
Woodruff CB3+ISCOM RD i.p.	0 (0/10)	128
Woodruff CB3+ISCOM RD s.c.	0 (0/13)	512

**Figure 3** Mortality after vaccination with live vaccine and the ISCOM preparation, administered s.c. and i.p. —, Control CB3M; ····, RD; ---, ISCOM s.c.; — · —, ISCOM, i.p.

sick around day 4 after infection with this virus. Many die suddenly (probably from heart arrhythmias). After 7 days massive myocarditis and also pancreatitis is encountered¹⁵. Those who live longer get more and more sick, some appear paralytic. After 14 days very few mice survive (about 5%).

The resulting experimental mortality on day 12 is shown in *Table 1* and the survival curve is depicted in *Figure 3*. The RD virus itself causes a very low mortality and can be regarded as an attenuated strain. The myocarditic CB3 caused roughly 78% mortality by day 12. All vaccinated animals survived if immunized with a dose of 0.65 µg and boosted once (*Table 1*). In polio vaccination a titre of eight is regarded as protective. Using the ISCOM preparation s.c. (512) a 16-fold titre elevation over the control animals (≤32) is seen (*Table 1*). This would be 64 times the protective level. A titre rise of four is considered significant so ISCOM vaccination is significantly raising the titre. Infection with the 'attenuated' strain CB3RD raises the titre twofold, i.e. not significant. CB3RD infected animals have a low mortality and a neutralization titre 1/8 of s.c. ISCOM vaccinated.

Minimal effective dose

To find out the smallest protective dose of vaccine polypeptide a dilution series was performed. As shown

(*Figure 4*) 16 ng administered three times s.c. conferred total protection. A dose of 1.6 ng gave partial protection. Even at 0.16 ng a delayed mortality was observed as compared to challenge infected animals.

Pathology

The histology showed extensive infiltration in control infected hearts (*Figure 5*). Inflammatory lesions and necrosis covered 5.7% of the myocardial area. No infiltrate could be found in animals vaccinated with 16 ng (*Figure 6*).

Immune histology showed a picture similar to previously published distribution of lymphocyte subsets, i.e. mainly L3T4⁺ (T-helper) cells¹⁵. Also suppressor-cytotoxic T cells, B cells and Ia-positive cells were found. No such cells were found in vaccinated animals (16 ng administered three times).

Discussion

Subunit vaccines have proven successful and are in current clinical use for hepatitis B (using HBsAg) and influenza (NA and HA). Other current viral vaccines are based on either live attenuated strains (mumps, morbilli, rubella, yellow fever and the Sabin oral polio vaccine) or inactivated virus. Inactivation is accomplished by

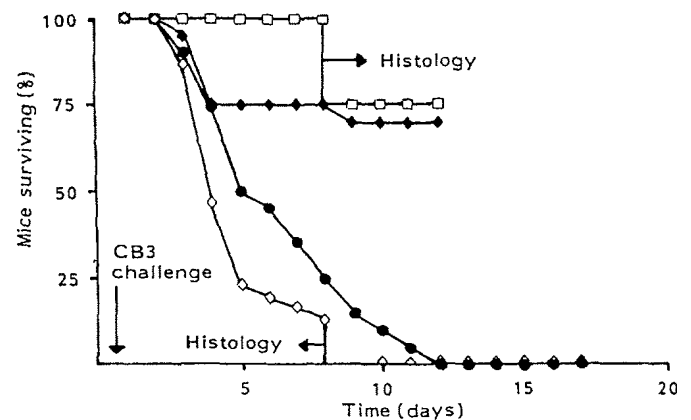
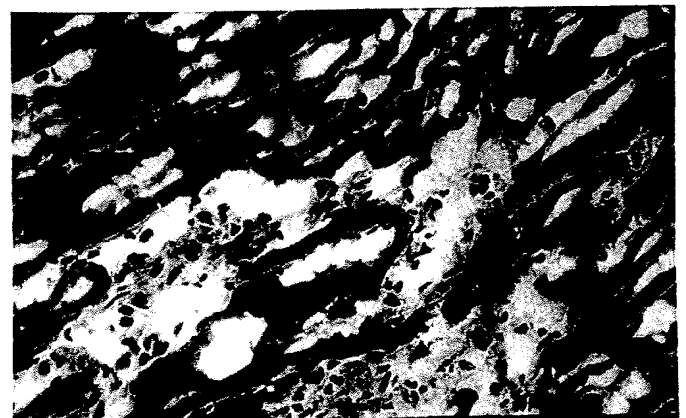
**Figure 4** Dose-response relationship of ISCOM vaccine preparation. Three doses were employed, administered three times: ◇, Control; ●, 0.16 ng; ◆, 1.6 ng; □, 16 ng. Four mice were taken out for histology day 8**Figure 5** Histology of Balb/c mouse heart after CB3M infection (without previous vaccination)



Figure 6 Histology of Balb/c mouse heart after ISCOM CB3-RD vaccination scheme and challenge CB3M infection

formalin (the Salk polio vaccine, influenza) or β -propiolactone (rabies). Many subunit vaccines are presently being experimentally tested. Among them are the so-called peptide vaccines. They are based on the idea that only the antigenic site is needed for induction of protective immunity. Subunit vaccines offer the advantage that nucleic acid can be avoided and thereby no chance for unwanted mutations exists. In this study efforts were not specifically made to free the preparation from RNA. The main purpose was to explore the possibility of ISCOM to contain antigens from a non-enveloped virus and consequently to induce protective immunity. Furthermore, subunit vaccines offer the possibility to purify the virus and its subunits, from contaminating cell culture proteins. Thus, the risk of allergic vaccine reactions is minimized.

In the present study a subunit ISCOM vaccine consisting of the three virus proteins VP1, VP2 and VP3 induced complete protection in such a low dose as 16 ng given three times. The route of administration, i.e. intraperitoneal or subcutaneous, seemed not to be important for protection. Even as low a dose as 0.16 ng delayed death from the challenging infection. The virus neutralization test confirmed that the ISCOM concept seems to offer good immunostimulant properties, ensuring high levels of neutralizing antibodies. In fact, also a cellular response might be stimulated as shown in the case of influenza¹⁶ as well as CMV¹⁷.

There are several reasons to select a subunit vaccine as has been mentioned above. Microorganisms possibly use a strategy to hide essential protective antigenic determinants. By rearrangement of subunit structures in the ISCOM, the microbe's defence might be evaded allowing protective immunity to be induced¹⁸. In the case of Coxsackie B3, it is likely that potentially protective antigens are hidden in 'canyons' formed by both VP1 and VP2¹⁹. In an ISCOM VP1 and VP2 are separated from each other. Thus, epitopes hidden in the canyon structure are exposed. The conditions created to split the virus (6M urea and SDS) are harsh, but apparently did not denature the neutralizing antigenic determinants. Maybe hidden determinants revealed by rearrangement are more conserved. If so, broader protective immunity might be induced than with a conventional vaccine.

Furthermore, the ISCOM vaccine technology might be of great interest for picornaviruses in general with their many serotypes.

Acknowledgements

Financial support was provided by Svenska Diabetesförbundet, Swedish National Association against Heart and Chest Diseases, Svenska livförsäkringsbolags nämnd för medicinsk forskning and the Family Ernfors Fund. The authors are grateful to: Dr Mikael Lindberg for help with virus preparation; Dr Neal Barnes for help with gel electrophoresis; Dr Osterhaus for serologic analysis; and Dr Lars Wesslén for help with computer technology.

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